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β-Lactam Resistance in *Staphylococcus aureus* Cells That Do Not Require a Cell Wall for Integrity

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Staphylococcus aureus **ATCC 9144 cells with defective cell walls were generated on a medium with elevated osmolality in the presence of sublethal levels of penicillin G. On removal of antibiotic pressure, the cells exhibited stable penicillin and methicillin resistance. The resistance was homogeneous and its acquisition was enhanced following transient cell wall-defective growth. The resistant cells were *mecA* negative, β-lactamase negative and did not contain any mutations in the coding regions of *pbp* genes. When penicillin was added back to resistant cells, they continued to grow and produced a diffuse cell wall that was resistant to the action by lysostaphin but was very sensitive to lysis with Triton X-100. These data indicate that the resistant cells are not dependent upon an intact cell wall for osmotic stability and they are able to switch readily to this mode of growth in the presence of penicillin G.**

Originally called L-forms, cell wall-defective (CWD) bacteria were first described in *Streptobacillus moniliformis* (11). We now know that many bacteria can become cell wall defective in the presence of various agents, including cell wall-active antibiotics, and can be propagated indefinitely on suitable media. CWD bacteria have been reported under a number of conditions, including burn site infections (10), sarcoidosis (5), and culture-negative febrile episodes in bone marrow transplant patients (15). Indeed, the clinical importance of CWD bacteria may be underestimated, as they do not grow on routine bacteriological media and are resistant to antibiotics that act on the cell wall. Moreover, cell wall-defective variants of *Staphylococcus aureus* were shown to be ingested by rat peritoneal macrophages, without phago-lysosomal fusion and digestion (12), and should therefore be expected to evade the immune system by intracellular refuge.

In the presence of antibiotics, transient cell wall deficiency could also give bacteria time to develop stable antibiotic resistance. This idea is supported by a recent observation that the probability of obtaining β-lactamase-derepressed mutants of *Enterobacter cloacae* rose dramatically when the bacteria were incubated on a medium supporting the growth of CWD bacteria in the presence of ticarcillin (9).

In this paper we discuss an adaptive response of bacteria exposed to antibiotic pressure that has been frequently observed but little understood and its role in facilitating antibiotic resistance. We have demonstrated that transient penicillin-induced loss of the cell wall in *S. aureus* mediates high-level resistance to β-lactam antibiotics and that following recovery of the cell wall, the penicillin resistance is inherited in a stable manner. Furthermore, cells that had previously been cell wall defective had a propensity to lose their cell wall on further penicillin exposure.

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MATERIALS AND METHODS

Bacterial strains and growth. *Staphylococcus aureus* ATCC 9144 was used as a source of cell wall variants because it was β-lactamase negative and susceptible to penicillin (MIC, 0.015 mg/liter in broth dilution tests using CWD medium). *S. aureus* ATCC 25923 was used as an additional control for antibiotic susceptibility testing. Strains were maintained on Luria-Bertani medium (10% [wt/vol] Bacto Tryptone, 5% [wt/vol] Bacto-yeast extract, 10% [wt/vol] NaCl [pH 7.5]). Medium for the induction and growth of cell wall-defective variants (CWD medium) consisted of brain heart infusion broth (Oxoid, Basingstoke, United Kingdom) supplemented with sucrose 5% (wt/vol), yeast extract 0.5% (wt/vol), horse serum 10% (vol/vol), magnesium sulfate 0.2% (wt/vol). Penicillin G was included, at various concentrations, as described. The medium had an osmolality of 597 mosmol/kg as measured by the Advanced Micro-osmometer (model 3MO plus) using Clinitrol 290 as the standard. Where necessary, media were solidified by the addition of agar (1% [wt/vol]). Total cell counts were carried out on blood agar or CWD medium without added antibiotics.

Generation of cell wall-defective bacteria. *S. aureus* ATCC 9144 was streaked onto CWD medium in the presence of various concentrations of penicillin G and incubated at 37°C in air. The plate with the highest concentration of penicillin G that could support growth showed colonies with atypical morphology from which the cells stained gram negative. Cells growing at this penicillin concentration were picked and restreaked upon fresh plates containing further incremental increases in the antibiotic concentration.

CWD bacteria could also be efficiently induced in liquid medium of the same formulation; typically, 5-ml cultures were grown aerobically at 37°C with continuous agitation.

Antibiotic susceptibility testing. Bacteria were grown overnight at 37°C in air on CWD agar, supplemented with penicillin as appropriate. A suspension of each organism that gave semiconfluent growth was used for antibiotic susceptibility testing, which was performed according to the British Society for Antimicrobial Chemotherapy guidelines for disk diffusion, broth dilution, and E-tests (1). For disk-diffusion tests, disks contained either penicillin (1 μg) or oxacillin (1 μg). No differences were observed in zone sizes in the disk-diffusion test, for either *Staphylococcus aureus* ATCC 9144 or ATCC 25923, on CWD medium and on Iso-Sensitest agar.

Population analysis. The distribution of antibiotic resistance in cell populations was determined by spotting undiluted or appropriately diluted aliquots

(10 μl) onto duplicate CWD plates containing various concentrations of penicillin G. Plates were incubated at 37°C for 48 h before the colonies were enumerated.

Electron microscopy. Samples for transmission electron microscopy were prepared from cultures grown in CWD broth supplemented with penicillin as required. The cells were collected by centrifugation and fixed in 0.1 M sodium cacodylate buffer, 4% (vol/vol) paraformaldehyde, 2.5% (vol/vol) glutaraldehyde (pH 7.4). After 2 h cells were postfixed in cacodylate-buffered 1% (wt/vol) osmium tetroxide. After dehydration in a graded series of ethanol and propylene oxide, the bacterial cells were embedded in Araldite CY212. Thin sections (70 to 90 nm) were stained with uranyl acetate and lead citrate before examination using a Philips 400T microscope operating at 100 kV.

Sequencing of *pbp* genes. PCR primers, for each gene, were synthesized from consensus alignments of published *S. aureus* genome sequences. The primers were as follows: pbp1 sense primer, 5′-GATACGCGAGGAAAGATTGC-3′; pbp1 reverse primer, 5′-TTTACGGCATAAGAGGCCAG-3′, which produced a PCR product of 2,596 bp in length, including 172 bp of 5′ untranslated region (UTR) and 189 bp of 3′ UTR; pbp2 sense primer, 5′-TCGAAGTATTTTGGA AGAG-3′; pbp2 reverse primer 5′-GTGAATGACTGATTTTACG-3′, which produced a 2,504-bp product that included 160 bp of 5′ UTR and 163 bp of 3′UTR; pbp3 sense primer, 5′-GTATGATTACTTGTTCGGTCTC-3′; pbp3 reverse primer, 5′-CAACCATGCGCTACACAATC-3′, which produced a 2,228-bp product, including 119 bp of 5′ UTR and 33 bp of 3′ UTR; pbp4 sense primer, 5′-GAGTAAGTTTGCTCTTCG-3′; pbp4 reverse primer, 5′-GTACAG AAGGCATTTCGACG-3′, which produced a 1,679-bp product with 205 bp of 5′ UTR and 178 bp of 3′ UTR.

PCR was carried out for 30 cycles, with each cycle consisting of 94°C for 45 s, 50°C for 45 s, and 72°C for 2 min using KOD polymerase (Novagen). The PCR products were purified using the QIAquick PCR purification system (QIAGEN) and eluted in a volume of 50 μl. A-tails were added to purified PCR products using *Taq* polymerase in the presence of 0.3 mM dATP at 72°C for 10 min. The PCR products were ligated into the vector pCR TOPO TA (Invitrogen) and transformed into *Escherichia coli* XL1-Blue (Stratagene), and recombinant plasmids were selected on plates containing ampicillin and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

DNA was prepared for sequencing from overnight liquid cultures using the Wizard Plus SV DNA purification system (Promega) and was sequenced with an Applied Biosystems 373A DNA sequencer using a primer walking approach. Both strands of each gene were completely sequenced, and contiguous sequences were constructed using DNA Strider computer software.

PBP binding assays. Penicillin-binding protein (PBP) binding assays were carried out according to the method of Tonin and Tomasz (14) on purified membrane fractions. Membrane protein concentrations were estimated using the DC protein assay kit (Bio-Rad) using bovine serum albumin as standard. Labeled PBPs were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% gel and visualized by fluorography. PBPs on the autoradiograph were quantified using NIH Image v1.63 software.

Lysostaphin and Triton X-100 lysis assays. Cells were grown to mid-exponential phase in liquid medium, washed three times in 20 mM potassium phosphate (pH 7.5), and resuspended in the same buffer to an optical density at 620 nm of approximately 0.25. For lysostaphin assays, 1.0 ml of suspended cells was placed into a spectrophotometer at 37°C, and the change in absorbance was recorded continuously following the addition of 1 U of lysostaphin. For Triton X-100 assays, 10 ml of suspended cells was placed in a sterile 25-ml Erlenmeyer flask in a shaking water bath set at 37°C, and Triton X-100 was added to a final concentration of 0.1% (vol/vol). Samples were taken at intervals to monitor the change in absorbance. In both cases values were adjusted to a percentage of the initial absorbance.

Nucleotide sequence accession numbers. The nucleotide sequences determined in the present study have been deposited in GenBank and have the following accession numbers: *pbp1*, AY920399; *pbp2*, AY920400; *pbp3*, AY920401; *pbp4*, AY920402.

RESULTS

Cell wall-defective bacteria grow in the presence of penicillin. CWD variants of *S. aureus* were generated in a medium with elevated osmotic potential (597 mosmol/kg) in the presence of sublethal levels of penicillin G. *S. aureus* ATCC 9144 was incubated aerobically for up to 72 h on media containing a range of concentrations of penicillin G. Approximately 1 in 10,000 of the cells inoculated onto a plate containing half the MIC of antibiotic (0.0075 mg/liter) gave rise to colonies with a cell wall-defective phenotype. The colonies were small, had lost the yellow pigmentation typical of *S. aureus*, and were umbonate in profile. On Gram staining, the cells had indistinct margins, did not show typical staphylococcal cell arrangements, and were gram negative.

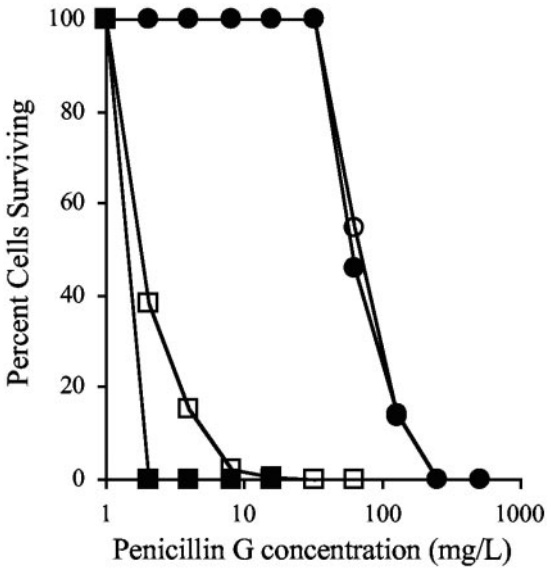

FIG. 1. Population analysis of *S. aureus* cells grown in the presence of penicillin on medium that either induced cell wall-defective variants (CWD medium) or did not (LB medium). CL3/19 (open circles), cells grown in CWD medium in the presence of penicillin G; RL3/19 (closed circles), CL3/19 cells passaged in CWD medium in the absence of penicillin G five times; LB9 (open squares), cells grown in LB medium in the presence of penicillin G; LB9/2 (closed squares), LB9 cells passaged in LB medium in the absence of penicillin G five times.

Cell wall-defective variants could be maintained by subculture on CWD medium containing penicillin. Transfer of the cells to blood agar, LB medium, or CWD medium without addition of penicillin caused them to revert to cell wall-competent (CWC) forms that stained gram positive (data not shown).

Acquired penicillin resistance is stable in CWC cells. The penicillin concentration was increased incrementally in two independent experiments to generate cell wall-defective variant lines on solid medium (CS1/19) and in liquid medium (CL3/19) that were able to grow in the presence of penicillin G at a concentration of 19.2 mg/liter. In order to determine whether the penicillin resistance phenotype of the CWD variants was due solely to loss of the antibiotic target, cells were then allowed to revert to CWC forms prior to antibiotic susceptibility testing by subculturing on CWD medium or LB medium without antibiotic. Following a single passage in the absence of antibiotic, CWC cells (designated RS1/19/1) stained uniformly gram positive. When tested by antibiotic disk diffusion, both CS1/19 and RS1/19/1 strains were resistant to penicillin G and oxacillin, whereas the parental strain, *S. aureus* ATCC 9144, produced large zones of inhibition (zone diameters, 32 mm ± 1.6 mm for penicillin G and 29 mm ± 1.4 mm for oxacillin). In view of the fact that RS1/19/1 might contain a

heterogeneous population, it was then subcultured 10 times on antibiotic-free medium, producing strain RS1/19/10, which was retested with the two β-lactam antibiotics. These cells also stained gram positive and remained completely resistant to the penicillin G- and oxacillin antibiotic-containing disks. Similar results were obtained when either LB or CWD medium was used for antibiotic-free growth. These results showed that the CWC cells had stable resistance to β-lactam antibiotics.

Acquisition of penicillin resistance is enhanced in cell wall-defective bacteria. To determine the extent to which stable high-level penicillin resistance was due to loss of cell wall integrity, *S. aureus* ATCC 9144 cells were exposed to sublethal levels of penicillin G in two different liquid media. One (CWD medium) permitted the cell wall-defective phenotype, and the other (LB medium) did not. Following nine serial passages in increasing concentrations of penicillin G (0.5-fold to 128-fold times the original MIC), the population profiles of the strain grown in CWD medium (CL3/19) and the strain grown in LB medium (LB9) were determined with various concentrations of penicillin G (Fig. 1). In this way the proportions of resistant cells and the maximum level of resistance within each population were determined.

The maximum concentration of penicillin G that LB9 cells were subjected to during the nine serial passages was 9.6 mg/liter. Throughout the experiment, these cells stained uniformly gram positive, indicating that there was no cell wall disruption. Population analysis determined that 0.45% of the cells were able to grow at 9.6 mg/liter of antibiotic, and the maximum concentration at which these resistant cells grew was 38.4 mg/liter.

In contrast, cells grown in CWD medium (CL3/19) showed extensive gram-negative staining early in the experiment, indicating establishment of a cell wall-defective phenotype. The maximum concentration of antibiotic that these cells were subjected to was 19.2 mg/liter, and analysis showed that 100% of the cells in the population were able to grow at that concentration of antibiotic. The maximum concentration of penicillin G that these cells were able to tolerate was 76.8 mg/liter, and this degree of resistance was shown by 13.5% of the population. Clearly, the level of resistance to penicillin and the extent of resistance within the population were dramatically enhanced when cells had defective cell walls.

The stability of penicillin resistance in the two cell populations was tested following a total of five serial passages on the same media in the absence of antibiotic (Fig. 1). The strain that was grown in LB medium (LB9/2) showed a lower resistance to penicillin. Approximately 1 in 10^6 cells in this population was able to grow in the presence of 9.6 mg/liter penicillin G, the maximum concentration at which growth was recorded, showing that resistant cells had been lost from the population in the absence of antibiotic selection pressure. In contrast, 100% of cells in the strain that had been cell wall defective and subsequently recovered cell walls (RL3/19) were resistant to 19.2 mg/liter penicillin G, and the maximum concentration at which growth was recorded was 307.2 mg/liter. The transient loss of cell wall integrity had therefore enabled the cells to develop homogeneous, stable, high-level penicillin resistance. E-tests and broth dilution MIC determinations confirmed that the CWD strain CL3/19 and its CWC derivative (RL3/19) are resistant to high levels of penicillin G (Table 1).

TABLE 1. Penicillin G MICs of strains, determined by E-tests and broth dilution

MIC test/medium	MIC (mg/liter)				
	Wild type (ATCC 9144)	CL3/19	RL3/19	LB9	LB9/2
E-test/CWD	<0.016	192	>256	16	1.5
E-test/Iso-Sensitest	<0.016	192	>256	16	0.75
Broth dilution/CWD	0.016	64	128	ND	ND

[a] ND, not determined.

CWC cells showed no difference in susceptibility to vancomycin, which is a cell wall-active antibiotic with a different target site to that of β-lactams, compared to wild-type cells (MIC, 2 mg/liter). However, following growth of CWC cells in the presence of penicillin and concomitant changes to the cell wall structure, they had a decreased susceptibility to vancomycin (MIC, 512 mg/liter).

CWC cells exhibit *mecA*-negative, β-lactamase-negative methicillin resistance. CWC cells were tested for their susceptibility towards oxacillin, using E-test strips, and were found to have a MIC of 192 mg/liter. In order to establish the cause of this resistance, a series of investigations were undertaken to determine the presence or absence of known resistance mechanisms in these cells.

A duplex PCR using primers designed to amplify the *mecA* gene (3) and the *Staphylococcus*-specific *nuc* gene (4) showed that the *mecA* gene was absent. The cells were also tested with the Beta Test kit (Medical Wire and Equipment Co., Bath, United Kingdom) and were negative for β-lactamase activity. Furthermore, incubation of exponentially growing CWC cells with penicillin G did not inactivate the antibiotic (data not shown), confirming the absence of β-lactamase activity and excluding the presence of another antibiotic-inactivating activity.

Alterations in the coding region of penicillin-binding proteins, notably PBP2, have been implicated in non-PBP2a-mediated methicillin resistance (7). To investigate this possibility, the four *pbp* genes were cloned and sequenced from both wild-type *S. aureus* ATCC 9144 and the penicillin- and methicillin-resistant CWC strain RS3/19. No differences in the DNA sequence of any of the PBP genes were identified between the methicillin-sensitive wild-type strain and the methicillin-resistant CWC derivative. The lack of mutation in the coding and adjacent regions shows that the observed resistance is not due to alterations in the affinity of PBPs for penicillin or in regulatory elements immediately upstream of the genes.

To confirm that the CWC strain did not contain PBPs with reduced affinity for penicillin, 35 μg of membrane proteins from wild-type and CWC strains was incubated with [^{3}H]benzylpenicillin according to the method of Tonin and Tomasz (14). Separation of the membrane proteins by SDS-PAGE and fluorography showed that the CWC strain had increased binding of penicillin to PBP4 (Fig. 2). Densitometric analysis of the penicillin-labeled bands showed that the amount of binding to PBP1, -2, and -3 was similar in wild-type and CWC membrane proteins, whereas binding to PBP4 in the CWC strain was

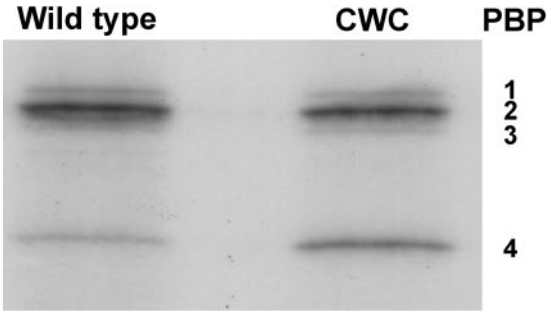

FIG. 2. Binding capacity of PBPs of a penicillin-resistant CWC line and its parent strain for benzylpenicillin. Membrane preparations were exposed to [3H]benzylpenicillin and separated by SDS-PAGE prior to fluorography.

2.75-fold greater than in the wild type. This greatly alters the ratio of penicillin-binding capacity between PBP2 and PBP4 from 4.6 in the wild type to 1.5 in CWC membrane proteins. As no mutations were detected in any *pbp* genes, the increased penicillin binding is likely to be due to an increase in the amount of PBPs in the membrane. This contention is supported by an observation from DNA microarray analysis that *pbp4* mRNA expression is 18-fold higher in CWC cells than in wild-type cells, whereas the other *pbp* genes are expressed at comparable levels in these two strains (T. Fawcett, unpublished data).

CWD variants do not rely upon peptidoglycan for cell integrity. Transmission electron microscopy of ultrathin sections demonstrated that cells grown in the presence of sublethal levels of penicillin G had a disorganized and incomplete cell wall and many cells had more than one division plane. The splitting system (for a review of staphylococcal cell wall morphogenesis, see reference 6) was absent from the cross wall of dividing cells, there was excessive production of wall material, and the margins of cells were indistinct. In contrast, the periphery of each wild-type *S. aureus* cell appeared as a compact structure with cross walls clearly showing the splitting system.

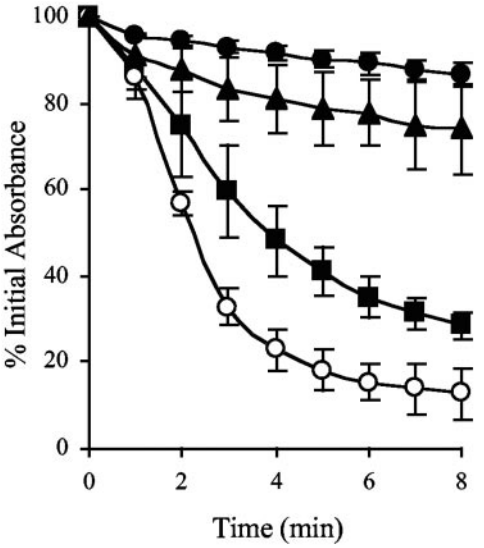

FIG. 4. Lysostaphin lysis of cells. Results shown are for lysostaphin treatment of wild-type cells (open circles), CWD cells (closed circles), CWC cells (closed squares), and CWC cells grown in the presence of penicillin (closed triangles).

On removing the antibiotic pressure, the appearance of CWC forms was indistinguishable from that of wild-type cells, but following growth in the presence of penicillin CWC cells showed major alterations to the cell wall structure. The walls of CWC cells grown in the presence of penicillin appeared similar to CWD cell walls, being thickened and diffuse and with evidence of more than one division plane in a single cell (Fig. 3).

In further experiments to explore the cell surfaces, two lysis methods were performed on bacteria grown in liquid medium: the first employed lysostaphin, a peptidase that cleaves the pentaglycine cross-bridge in staphylococcal peptidoglycan, and the second used the detergent Triton X-100. When wild-type cell suspensions were incubated in the presence of lysostaphin, rapid lysis was observed, which was followed spectrophoto-

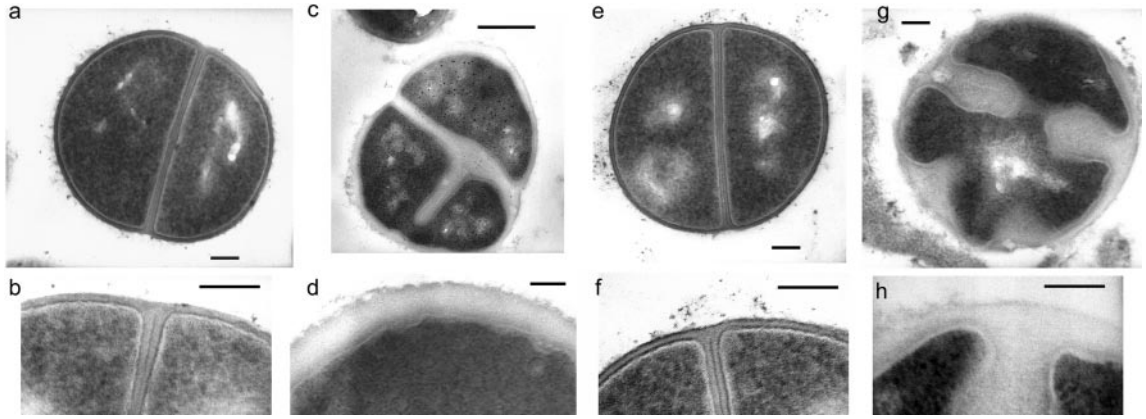

FIG. 3. Transmission electron micrographs of wild-type and cell wall-defective variants of *S. aureus.* (a and b) Wild-type cell wall appeared as an electron-dense structure at the periphery of the cell; nascent cell walls were also visible in dividing cells. (c and d) CWD cells showed a thickened and diffuse cell wall, with no visible splitting system and many cells having greater than one division plane. (e and f) The CWC strain had a cell wall structure visibly indistinguishable from wild-type cells. (g and h) On addition of penicillin to CWC cells, the cell wall became diffuse and cells had greater than one division plane. Bars, 0.1 μm.

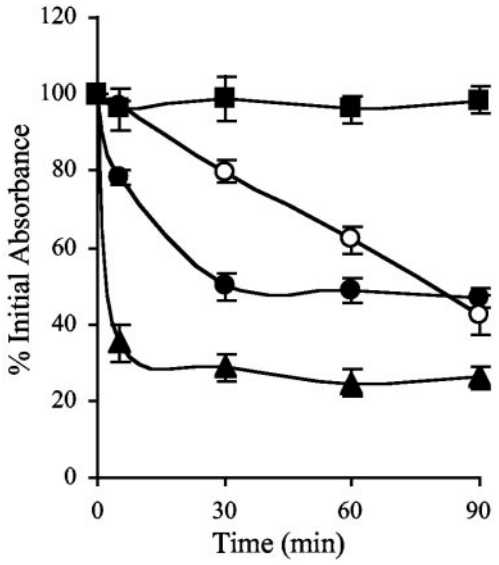

FIG. 5. Triton X-100 lysis of cells. Results shown are for Triton X-100 treatment of wild-type cells (open circles), CWD cells (closed circles), CWC cells (closed squares), and CWC cells grown in the presence of penicillin (closed triangles).

metrically at 620 nm (Fig. 4). CWD cells, however, did not show an increase in the rate of lysis following addition of lysostaphin and are thus not reliant on the presence of peptidoglycan for their intactness. On addition of lysostaphin to the CWC cell line, RL3/19, a decrease in absorbance was observed, demonstrating that these cells have an intact peptidoglycan structure that is integral to their intactness. CWC cells were slightly more resistant to the action of lysostaphin than wild-type cells, suggesting that there may be alterations in the peptidoglycan structure in this line. This change in lysostaphin susceptibility may be due to alteration of the target site for lysostaphin or to an increase in the number of pentaglycine cross-links in the peptidoglycan. Lysostaphin treatment of CWC cells that had been grown in the presence of penicillin caused a slow loss of absorbance with similar kinetics to that shown by CWD cells.

In contrast to lysostaphin treatment, CWD cell suspensions lysed rapidly when incubated with 0.1% Triton X-100, presumably because their membranes were more accessible to the detergent than were those of wild-type cells. CWC cells were resistant to lysis by Triton X-100, indicating that the recovery of a cell wall was impeding access of the detergent to the cell membrane. However, when grown in the presence of penicillin, CWC cells were highly sensitive to lysis by Triton X-100 (Fig. 5).

CWD cells have an altered cell wall appearance, and their resistance to the action of lysostaphin and their increased susceptibility to Triton X-100 show that CWD bacteria do not rely on an intact peptidoglycan structure for their integrity. This phenotype is mimicked only when CWC cells are grown in the presence of penicillin.

DISCUSSION

Cell wall-defective variants of *S. aureus* ATCC 9144 were formed readily on complex media with a high osmolality in the presence of the cell wall-active antibiotic penicillin G. The resulting colonies had an unusual appearance, including loss of the golden pigmentation, and lacked an organized cell wall structure. The CWD bacteria were resistant to β-lactam antibiotics, and this resistance persisted in a stable manner when cells were allowed to regain their cell wall integrity following subculture in the absence of antibiotic. The acquisition of high-level, stable resistance was only observed in those cells that had been cell wall defective and not in control cells grown on media that did not allow the formation of CWD bacteria. A similar phenomenon has been observed in the acquisition of derepressed β-lactamase mutants in *Enterobacter cloacae* (9). Population analysis showed that the resistance was present in 100% of the cells.

Resistance to β-lactam antibiotics in CWD bacteria was expected, but if this resistance had been due solely to loss of the antibiotic target, susceptibility to these antimicrobial agents should have been regained on recovery of the cell wall. However, following growth of CWC cells for many generations in the absence of antibiotics, the cells remained resistant to methicillin as well as penicillin G.

Decreased microbial susceptibility to β-lactam antibiotics has been widely reported, and a number of resistance mechanisms have been demonstrated. In staphylococci the most widely recognized mechanism is the *mecA* gene, which encodes PBP2a, a PBP2 variant with low penicillin-binding affinity. Resistant strains usually also contain an inducible β-lactamase. Borderline methicillin resistance has also been recognized in clinical isolates that do not contain either the *mecA* gene or a β-lactamase (2, 13) but have mutations in the *pbp* genes that cause reduced penicillin binding (7). Each of these mechanisms has been eliminated as an explanation for the resistance we observed in CWC cells. The increase in penicillin binding observed in CWC cells is likely to be due to alterations in the regulation of the gene rather than to mutations in the coding sequence. Overproduction of PBP4 has been previously reported to increase resistance to β-lactams, with the levels of methicillin resistance increasing from two- to sixfold in various strains (8). Though an increase in the level of PBP4 in cells contributes to methicillin resistance in a small way, this alone cannot account for the high-level resistance observed in CWC cells.

Uniquely, we have observed that after reintroduction of penicillin the integrity of CWC cells does not depend on their reconstituted cell walls. The CWC cells have a compact cell wall structure that responds to lysostaphin treatment in a manner similar to wild-type cells, with rapid cell lysis. However, on treatment with penicillin, CWC cell walls appear as diffuse structures, and the cells maintain their integrity in the presence of lysostaphin. CWD and CWC cells, grown in the presence of penicillin, are extremely sensitive to lysis by Triton X-100, likely due to increased access to the membrane because of the diffuse nature of the call wall.

Interestingly, CWC cells are not resistant to other classes of cell wall-active antibiotics unless they are first grown in the presence of penicillin. This suggests that their ability to switch to a mode of growth that does not rely upon the presence of a cell wall for survival may be β-lactam specific. Together, these data indicate that the cells have undergone stable genotypic changes that allow them to avoid the action of β-lactam anti-

biotics by quickly and uniformly dispensing with the need for an intact peptidoglycan sacculus for osmotic stability. Furthermore, cells with a defective cell wall become resistant to other cell wall-active antibiotics and are able to grow and divide. The ability of these cells to survive may be an important bacterial response to attack by cell wall-active agents.

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REFERENCES

1. **Andrew, J. M.** 2001. Determination of minimum inhibitory concentrations. J. Antimicrob. Chemother. **48:**5–16.
2. **Berger-Bachi, B., A. Strassle, and F. H. Kayser.** 1989. Natural methicillin resistance in comparison to that selected by *in vitro* drug exposure in *Staphylococcus aureus*. J. Antimicrob. Chemother. **23:**179–188.
3. **Bignardi, G. E., N. Woodford, A. Chapman, A. P. Johnson, and D. C. Speller.** 1996. Detection of the *mecA* gene and phenotypic detection of resistance in *Staphylococcus aureus* isolates with borderline or low-level methicillin resistance. J. Antimicrob. Chemother. **37:**53–63.
4. **Brakstad, O. G., K. Aasbakk, and J. A. Maeland.** 1992. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the *nuc* gene. J. Clin. Microbiol. **30:**1654–1660.
5. **El-Zaatari, F. A. K., S. A. Naser, D. C. Markesich, D. C. Kalter, L. Engstand, and D. Y. Graham.** 1996. Identification of *Mycobacterium avium* complex in sarcoidosis. J. Clin. Microbiol. **34:**2240–2245.
6. **Giesbrecht, P., T. Kersten, H. Maidhof, and J. Wecke.** 1998. Staphylococcal cell wall: morphogenesis and fatal variations in the presence of penicillin. Microbiol. Mol. Biol. Rev. **62:**1371–1414.
7. **Hackbarth, C. J., T. Kocagoz, S. Kocagoz, and H. F. Chambers.** 1995. Point mutations in *Staphylococcus aureus* PBP 2 gene affect penicillin-binding kinetics and are associated with resistance. Antimicrob. Agents Chemother. **39:**103–106.
8. **Henze, U. U., and B. Berger-Bachi.** 1996. Penicillin binding protein 4 overproduction increases β-lactam resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **40:**2121–2125.
9. **Huber, T. W.** 2002. Growth of cell wall deficient variants of *Enterobacter cloacae* facilitates beta-lactamase derepressed mutants. Int. J. Antimicrob. Agents **19:**397–404.
10. **Jiang, H. Q., Y. F. Chen, A. N. Li, and Z. D. Li.** 1994. Clinical burn wound infection caused by L-forms of *Staphylococcus aureus*. Burns **20:**83–84.
11. **Klieneberger, E.** 1935. The natural occurrence of pleuropneumonia-like organisms in apparent symbiosis with *Streptobacillus moniliformis* and other bacteria. J. Pathol. Bacteriol. **40:**93–105.
12. **Schmitt-Slomska, J., L. Michailova, E. Ivanova, and A. Toshkov.** 1986. Adhesion and phagocytosis of *Staphylococcus aureus* L-forms. J. Basic Microbiol. **26:**429–440.
13. **Tomasz, A., H. B. Drugeon, H. M. Delencastre, D. Jabes, L. McDougall, and J. Bille.** 1989. New mechanism for methicillin resistance in *Staphylococcus aureus* clinical isolates that lack the *pbp2a* gene and contain normal penicillin-binding proteins with modified penicillin-binding capacity. Antimicrob. Agents Chemother. **33:**1869–1874.
14. **Tonin, E., and A. Tomasz.** 1986. Beta-lactam-specific resistant mutants of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **30:**577–583.
15. **Woo, P. C. Y., S. S. Y. Wong, P. N. L. Lum, W. T. Hui, and K. Y. Yuen.** 2001. Cell-wall-deficient bacteria and culture-negative febrile episodes in bone-marrow-transplant recipients. Lancet **357:**675–679.